BIPHASIC MICROTITER METHOD FOR CAMPYLOBACTER RECOVERY AND ENUMERATION

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ABSTRACT

We developed a biphasic method for recovery and enumeration of campylobacters. The biphasic system was composed of 96-well microtiter plates containing Campy–Line agar prepared 2× for all ingredients except for 1× agar (0.1 mL per well). Samples were prepared in either twofold or fivefold dilution series across separate 96-well plates and directly transferred (0.1 mL) to the agar-containing microtiter plates to create the biphasic system. Biphasic plates were incubated for up to 48 h at 42C either aerobically or microaerobically in a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. Campylobacter was recovered in the aerobically incubated biphasic plates; however, significantly greater Campylobacter recovery was obtained in the biphasic plates incubated microaerobically ($P < 0.05$*). Significantly higher numbers of injured campylobacters from chicken carcass rinse samples were recovered by the biphasic method compared with conventional direct plating techniques (*$P < 0.05$*).*

INTRODUCTION

The association of *Campylobacter* spp. with poultry is well established (Hartog *et al*. 1983; Stern *et al*. 2001). The majority of raw poultry products are contaminated with *Campylobacter jejuni* (Blazer *et al*. 1983; Stern 1992). Because the prevalence of campylobacters associated with poultry is so high, more meaningful quantitative information is desirable. Methods for detecting and enumerating campylobacters are tedious and time-consuming. Detection of sublethally injured cells associated with food products or environmental

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samples may be especially troublesome. The environmental parameters encountered during traditional poultry processing (high and low temperature extremes, chlorine, an oxygen rich environment, etc.) are detrimental to the survival of campylobacters (Mead *et al*. 1995). Therefore, any campylobacters surviving on poultry carcasses after processing are likely to be in a stressed condition. These stressed campylobacters are a food-safety concern because when ingested by humans they may still be capable of causing subsequent infection and illness. Current cultural methods employing immediate exposure of samples to high levels of antibiotics to eliminate competing noncampylobacter organisms may inadvertently further stress or kill sublethally injured campylobacters present in the sample and give misleading negative results. There is a need for a sensitive method for detecting and enumerating all viable campylobacters (healthy as well as stressed cells) resulting from food processing or other environmentally stressful conditions adverse to the survival of the pathogen.

Biphasic techniques for recovery of campylobacters have been documented to provide more abundant growth of the organism than other methods relying upon selective broth or agar recovery alone (Rollins *et al*. 1983; Shadowen and Sciortino 1989). The nature of the enrichment process, however, allows most biphasic systems to be used only for detection but not for enumeration of the pathogen. The purpose of this study was to develop a method for enhanced recovery and enumeration of viable campylobacters associated with processed, raw broiler chicken carcasses by combining the enumerative capabilities of a microtiter dilution well assay with biphasic growth conditions.

MATERIALS AND METHODS

Preparation of Campylobacter Biphasic Enumeration Plates

Campylobacter biphasic enumeration plates (CBEPs) consisted of an agar layer containing selective antibiotics covered by an equal volume of liquid sample in sterile 96-well microtiter plates (Corning Inc., Nagog, MA). Campy–Line agar (CLA; Line 2001) was prepared 2× for all ingredients except for 1× agar that was added (0.1 mL per well) to the microtiter plates and allowed to solidify. For the initial studies, triplicate samples of pure *C. jejuni* isolates, post-chill commercial chicken carcass rinse or *C. jejuni*-spiked carcass rinse, were prepared in either twofold or fivefold dilution series using a sterile phosphate buffered saline solution (PBS) across separate 96-well dilution plates and directly transferred (0.1 mL) to the CLA-containing CBEP wells to create the biphasic system. CBEPs were incubated for up to 48 h at

42C either aerobically or microaerobically in a gas mixture of 5% O_2, 10% CO_2 and 85% N_2. Wells in CBEPs incubated aerobically were covered with 0.1 mL of sterile mineral oil prior to incubation: microaerobic CBEP wells were not covered. Results from the CBEPs were compared to standard direct surface-plating methods on CLA in petri dishes incubated similarly under microaerobic conditions. Enumeration was conducted by observing the color change in the biphasic wells due to the ability of campylobacters to reduce the colorless triphenyltetrazolium chloride (TTC) in the CLA to red-colored formazan compounds. Reduction of TTC to formazan compounds is not unique to campylobacters; however, the selective antibiotics in the agar serve to eliminate most non-*Campylobacter* microbes. Presumptive positive *Campylobacter* spp. growth was recorded by noting the formation of dark-red precipitate in the wells. Wells negative for growth did not change color. Presence of *Campylobacter* spp. was further confirmed in the most dilute wells demonstrating growth by observing a characteristic corkscrew motility in wet mounts examined using phase-contrast microscopy and testing of representative wells by latex agglutination (*Campylobacter* Test Kit, Oxoid, Basingstoke, England). Estimation of mean campylobacter populations from triplicate wells was accomplished by the method of Kang and Siragusa (2001). The reciprocal of the dilution of the endpoint or the last well with positive growth (defined by visible observation of the reduction of TTC to the red-colored formazan compounds) was used to calculate the minimum cell number possible for that dilution and transformed to the $\log_{10}$ cfu/mL. Alternatively, the positive wells from the triplicate samples were read as a three-tube most probable number (MPN) code similar to the work of Fung and Kraft (1969). Appropriate MPN estimations were then derived using the method and MPN calculator of Curiale (2004). Statistically significant differences between groups were determined by *t*-test analysis (SigmaStat, Jandel Scientific Software, San Rafael, CA).

Broiler Carcass Rinse Sample Analysis

Following optimization of the biphasic microtiter plate method, commercial, post-chill, post-drip, broiler chicken carcasses ($n = 28$) were individually rinsed in 100 mL buffered peptone using a carcass-shaking machine and held on ice for immediate analysis (Dickens 1985). A twofold dilution series (dilution plate) was prepared by dispensing 0.1 mL of sterile PBS into 11 of the 12 columns in a 96-well plate (the first column was left empty). Triplicate samples (0.2 mL) were then dispensed into individual wells in the first column of the dilution plate. Samples were diluted twofold across the plate using an eight-channel pipettor by transferring 0.1 mL from the first column to the adjacent column on the right with thorough mixing and continuing the process across the dilution plate. Aliquots (0.1 mL) from the dilution plate were then

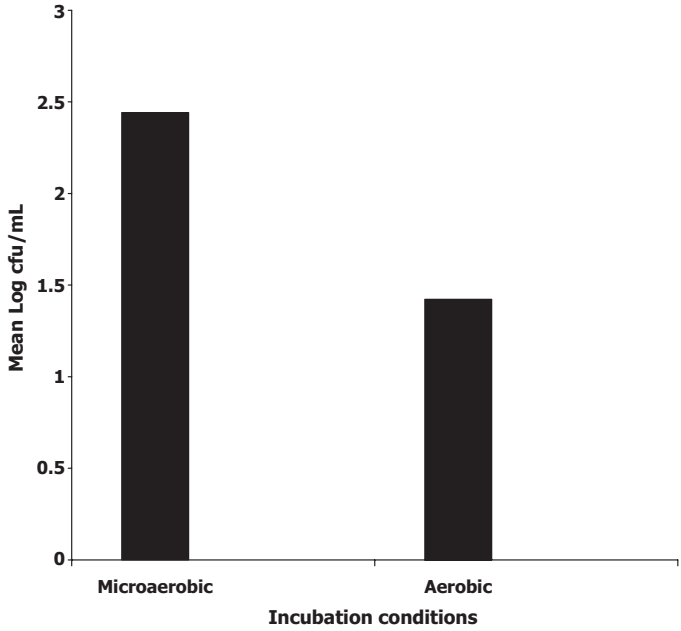

Fig. 1. ENUMERATION OF CAMPYLOBACTERS FROM RAW, PROCESSED CHICKEN CARCASSES ON BIPHASIC MICROTITER PLATES INCUBATED UNDER AEROBIC (WITH OIL OVERLAY) OR MICROAEROBIC CONDITIONS

directly inoculated into the corresponding wells on CBEPs prepared as before. The inoculated CBEPs were incubated under microaerobic conditions at 42C for 48 h. Samples were also surface plated on CLA plates (0.25 mL on each of four plates), which were similarly incubated for 48 h at 42C prior to enumeration.

RESULTS AND DISCUSSION

Microtiter plates incubated aerobically with an oil overlay did support *Campylobacter* recovery from all sample types (pure culture, spiked and unspiked carcass rinses); however, false positive wells (wells which turned red, but did not have characteristic *Campylobacter* spp. appearance upon microscopic examination) were occasionally observed. Significantly higher populations of *Campylobacter* ($P = 0.04$) were recovered from plates incubated under microaerobic conditions (Fig. 1; mean Log_{10} cfu/mL = 2.44 +/– 0.49) than those incubated under aerobic conditions (mean Log_{10} cfu/mL = 1.42 +/

– 0.91). While it is theoretically possible that non-*Campylobacter* organisms resistant to the specific antibiotics in CLA and present in the carcass rinse samples could give rise to false positive wells, no false positive wells were observed in plates incubated under microaerobic conditions. A twofold dilution series was found to give more accurate estimates of *Campylobacter* populations than fivefold dilutions do and was in a range suitable for estimating the populations of *Campylobacter* normally associated with chicken carcass rinse samples. *Campylobacter* populations estimated by determining the mean of three replicate samples demonstrating growth at the highest dilution and calculating the minimum cell number possible for that dilution were not significantly different than MPN values computed from similar replicate wells ($P = 0.66$).

Campylobacter populations associated with naturally contaminated, post-chill, post-drip, commercial broiler carcass rinse samples ($n = 28$) were determined using the optimal (twofold dilution, microaerobic incubation) CBEP method and direct surface plating on CLA. Again, significantly more campylobacter ($P = 0.02$) were enumerated by the biphasic microtiter method (Fig. 2; mean Log_{10} cfu/mL = 2.25 +/– 0.68) than by direct plating (mean Log_{10} cfu/mL = 1.79 +/– 0.74) and no false positive wells were detected by microscopic examination. It may be assumed that campylobacters present in carcass rinse samples have been stressed by commercial-processing conditions (i.e., temperature, chlorine, etc.). The incorporation of selective antibiotics into the solid agar layer of the biphasic microtiter system followed by the application of the liquid sample allows the antibiotic level to slowly increase in the liquid portion until equilibrium is achieved; thus allowing some recovery time for the injured cells. Biphasic recovery was efficacious for the sublethally injured organisms associated with this sample type presumably because of the slow release of selective antibiotics into the system. Slow or delayed addition of antibiotics is common to some *Campylobacter* recovery protocols (Corry *et al*. 1995). It might be possible to further optimize the biphasic system by incorporating different amounts of antibiotics in the agar layer, or by changing the concentration of agar present to alter diffusion times.

There are several advantages of the biphasic microtiter method for enumeration of campylobacters as compared to direct plating on selective agar. The technique is not complicated and can be rapidly conducted using a simple multichannel pipette. Because much smaller volumes of media are utilized, the biphasic microtiter system offers the advantages of reduced media costs while achieving increased recovery and enumeration of sublethally injured *Campylobacter*. The biphasic microtiter assay is a sensitive, cost-effective method for enhanced recovery and enumeration of healthy campylobacters as well as viable, sublethally injured campylobacters associated with raw poultry samples.

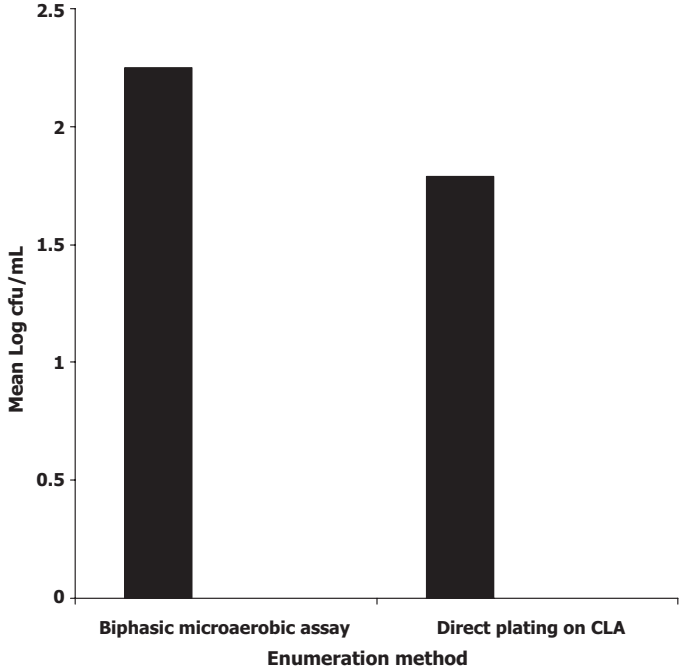

Fig. 2. ENUMERATION OF CAMPYLOBACTERS FROM RAW, PROCESSED CHICKEN CARCASSES BY DIRECT PLATING ON CAMPY–LINE AGAR (CLA) OR THE BIPHASIC MICROTITER METHOD

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